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Detection of human neutrophil elastase with peptide-bound cross-linked ethoxylate acrylate resin analogs

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Key words: cross-linked ethoxylate acrylate resin; chronic wound fluid; human neutrophil elastase; enzyme detection; peptide substrate; solid–liquid interface

Abstract: An assessment of elastase-substrate kinetics and adsorption at the solid–liquid interface of peptide-bound resin was made in an approach to the solid-phase detection of human neutrophil elastase (HNE), which is found in high concentration in chronic wound fluid. N-succinyl-alanine-alanine-proline-valine-*p*-nitroanilide (suc-Ala-Ala-Pro-Val-*p*NA), a chromogenic HNE substrate, was attached to glycine-cross-linked ethoxylate acrylate resins (Gly-CLEAR) by a carbodiimide reaction. To assess the enzyme-substrate reaction in a two-phase system, the kinetic profile of resin-bound peptide substrate hydrolysis by HNE was obtained. A glycine and di-glycine spacer was placed between the resin polymer and substrate to assess the steric and spatial requirements of resin to substrate with enzyme hydrolysis. The enzymatic activities of suc-Ala-Ala-Pro-Val-*p*NA and suc-Ala-Ala-Pro-Ala-*p*NA on the solid-phase resin were compared with similar analogs in solution.
An increase in visible wavelength absorbance was observed with increasing amounts of substrate-resin and enzyme concentration. Enzyme hydrolysis of the resin-bound substrate was also demonstrated on a polypropylene surface, which was employed for visible absorbance of released chromophore. A soluble active substrate analog was released from the resin through saponification of the ethoxylate ester linkages in the resin polymer. The resin-released conjugate of the HNE substrate demonstrated an increased dose response with increasing enzyme concentration. The synthesis and assay of elastase substrates bound to CLEAR resin gives an understanding of substrate-elastase adsorption and activity at the resin's solid–liquid interface for HNE detection with a solid-phase peptide.

Abbreviations: *p*NA, para-nitroaniline; CLEAR, cross-linked ethoxylate acrylate resin; DCM, dichloromethane; DMF,

dimethylformamide; DMSO, dimethylsulfoxide; FMOC, 9-flourenylmethyloxycarbonyl; Gly-CLEAR, glycine-cross-linked ethoxylate acrylate resins; HNE, human neutrophil elastase; suc-Ala-Ala-Pro-Val-*p*NA, N-succinyl-alanine-alanine-proline-valine-*p*-nitroanilide.

Introduction

Human neutrophil elastase is perhaps the most destructive enzyme in the body, and high human neutrophil elastase levels have been associated with a number of inflammatory disease states (1,2). An excessive concentration of elastase in chronic non-healing wounds has been shown to deleteriously degrade cytokine growth factors necessary for healing (3–5). On the contrary, it has been shown that minimal levels of elastase and other proteases which are found in acute wounds may also be required for an appropriate healing response. The potential to neutralize destructive elastase activity in the chronic wound either by controlled release (6) of an inhibitor or sequestration of elastase onto a dressing (7,8) suggests the necessity for a means of monitoring elastase levels in the chronic wound. Colorimetric detection of elastase in chronic wound fluid samples by a 'dip-stick method' would provide a means of monitoring and assisting in the neutralization of destructive elastase levels in the patient with a chronic wound.

Our approach to the understanding of detection of elastase activity on a solid phase is to immobilize an elastase substrate on a polymeric surface that serves as a matrix for enzymatic release of a chromophore absorbing at a visible wavelength (9). Cross-linked ethoxylate acrylate (CLEAR) resin supports (10) provide an interesting polymeric network to investigate the enzyme activity of immobilized substrates. Their ability to swell in both organic and aqueous solvents provides the potential for assessment of enzyme active substrates on resin supports following synthetic attachment of the enzyme substrate. This study reports the activity and enzyme-substrate interaction of human neutrophil elastase substrates containing para-nitroanilide (*p*NA) derivatives attached to CLEAR resin as an immobilized substrate assay to measure human neutrophil elastase activity.

Materials and Methods

Synthesis of elastase substrate-bound resin

The substrate bound CLEAR (Peptides International, Louisville, KY, USA) conjugates were synthesized with glycine and di-glycine as an amino-terminal linker. Amino-functionalized CLEAR-Base resin (HCl), 100–200 mesh was employed in the synthesis. The synthetic protocol for the synthesis of conjugate I on CLEAR resin consisted of the following steps. The resin was washed 3× with 20 mL of dimethylformamide (DMF) (5 min) and 3× with 20 mL of dichloromethane (DCM) (5 min). 9-flourenylmethyloxy-carbonyl-Gly-CLEAR (FMOC-Gly-CLEAR) resin was deprotected in 20% piperdine/DMF by shaking the mixture 3× in 15-min intervals. Thereafter, the deprotected resin was washed 3× with 20 mL of DMF (5 min) and 3× with 20 mL of DCM (5 min). N-succinyl-alanine-alanine-proline-valine-*p*-nitroanilide (suc-Ala-Ala-Pro-Val-*p*NA) (0.65 mmol) was coupled to Gly-CLEAR (0.1 g, 0.2 mmol/g) with diisoproplycarbodiimide (DIC) and 1-hydroxy-7-azabenzotriazole in DMF for 120 min. Thereafter, the modified resin was washed 3× with 20 mL of DMF (5 min) and 3× with 20 mL of DCM (5 min). Both the deprotection and the reaction-coupling steps were monitored with the Kaiser test (11). The modified resin was acetylated to cap unreacted glycine using (acetic anhydride : N-methylimidazole : DMF: 1 : 2 : 3). Synthesis of conjugate II on CLEAR resin was prepared with elastase substrate, suc-Ala-Ala-Pro-Ala-*p*NA, in the same manner as conjugate I. Samples were subjected to amino acid analyses. The resulting ratios of amino acids and peptide yields from the analyses are shown in Table 1.

Table 1. **Amino acid analyses of immobilized substrate on CLEAR**

		Ratio of amino acids			
Substrate	mmol/g	Gly	Ala	Pro	Val
CLEAR-Gly-suc-Ala-Ala-Pro-Val-*p*NA	0.102	1	2	1	1
CLEAR-Gly-Gly-suc-Ala-Ala-Pro-Val-*p*NA	0.028	3	2	1	1
CLEAR-Gly-suc-Ala-Ala-Pro-Ala-*p*NA	0.130	1	3	1	
CLEAR-Gly-Gly-suc-Ala-Ala-Pro-Ala-*p*NA	0.036	9	3	1	

Enzyme assay

The enzyme assay was conducted in pH 7.6 phosphate buffer [0.2 M sodium phosphate, 0.5 M NaCl, and 6.6% dimethylsulfoxide (DMSO)] with 5 μg/mL of elastase from human leucocytes (Sigma, St. Louis, MO, USA). Typically, 1–5 mg of substrate resin was placed in 90 μL of buffer. The enzymatic hydrolysis of the immobilized substrate conjugates I and II yield release of *p*NA monitored at 405 nm with incubation at 37 °C on a Bio-Rad Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA).

Microscopy

The swelling properties of Gly-CLEAR and CLEAR-Gly-suc-Ala-Ala-Pro-Val-*p*NA were investigated using an Olympus Model SZH Wide Field Stereo Microscope (Olympus Corp., Lake Success, NY, USA). Separate resin samples were swelled in phosphate buffer and DMSO for 5 min, and compared with dry resin at a magnification (β) of 72-fold on a microscopic slide.

Enzyme assay for substrate-resin analogs

Enzymatic substrate was cleaved from CLEAR resin by hydrolyzing the substrate resin (20, 30 and 40 mg) with 80 μL of 2 M NaOH buffer. The substrate resin was allowed to react for 2 h before centrifuging through a filtered vial. In a 96-well microtiter plate, 30 μL of elastase (Athens Research & Technology, Athens, GA, USA) was placed in the well and incubated for 10 min at 37 °C. Thereafter, 40 μL of supernatant was added to the enzyme and the reaction was monitored on a Microplate Reader (Bio-Rad). The release of *p*NA from the substrate was monitored at 405 nm with incubation at 37 °C on a Bio-Rad Microplate Reader.

Enzyme assay on polypropylene surface

An assessment of enzyme activity of the substrate resin on a surface designed for visible detection of chromophore release was accomplished by assaying the substrate resin on a white polypropylene surface. The assay was prepared by placing approximately 20 mg of CLEAR-Gly-suc-Ala-Ala-Pro-Val-*p*NA and approximately 20 mg of CLEAR-Gly-suc-Ala-Ala-Pro-Ala-*p*NA in individual wells of a Whatman® Uni-Filter® microplate (Whatman, Clifton, NJ, USA). The Uni-Filter® microplate has a melt-blown polypropylene filter media with a white background in which the chromophore absorbance can be monitored. In microtiter plate wells containing modified resins, 200 μL of human neutrophil elastase (20 μg/mL; Athens Research & Technology)

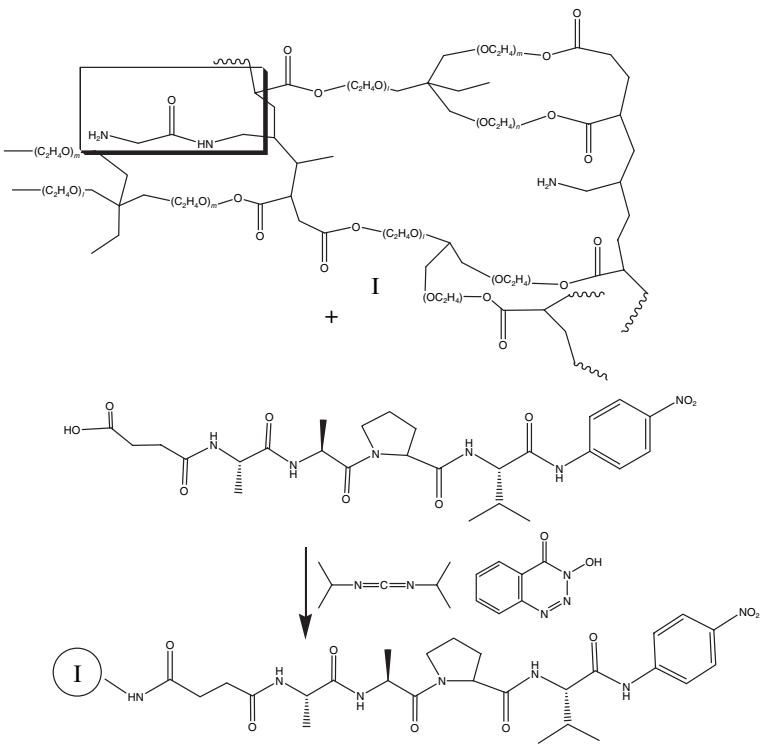

Figure 1. Structures of Gly-CLEAR resin I, the substrate analog suc-Ala-Ala-Pro-Val-*p*NA, and suc-Ala-Ala-Pro-Val-*p*NA linked to Gly-CLEAR resin through attachment of the amino-terminal succinyl group with the α-amino group of the glycine-bound CLEAR resin. The enzyme substrate suc-Ala-Ala-Pro-Val-pNA was reacted with Gly-CLEAR resin (glycine reactive site of resin is boxed in) through hydroxybenzotriazole-mediated carbodiimide coupling reaction in dimethylformamide as outlined in the Materials and Methods section. The substrate analogs in the text are indicated by the valine residue which is amino terminal to the scissle paranitroanilide with conjugate I as valine (R = propyl) and alanine (R = methyl) as conjugate II.

in phosphate buffer was added for interaction with substrate on the resin at 405 nm. The solid–liquid phase absorbances of modified resin were obtained at 0, 5, 10, 30 and 60 min while incubating at 37 °C.

Results

Synthesis and elastase activity of CLEAR-bound peptides

CLEAR resins were modified with the chromogenic elastase substrates suc-Ala-Ala-Pro-Ala-*p*NA and suc-Ala-Ala-Pro-Val-*p*NA to assess the colorimetric response of the immobilized substrate to human neutrophil elastase activity. The elastase substrate was attached to Gly-CLEAR resin through the amino-terminal succinyl carboxylate of the peptide derivative using conventional solid-phase synthesis techniques. The synthetic scheme of the substrate-resin preparation is shown in Fig. 1. Amino acid analyses of the peptide-resin products revealed from 0.028–0.130 mmol substrate per gram of resin (see Table 1). Amino acid ratios based on the analysis demonstrated a good reaction of the peptide substrates with the CLEAR. A sevenfold excess of unreacted glycine however was apparent with the analog CLEAR-Gly-Gly-suc-Ala-Ala-Pro-Ala-pNA. Acetylation of unreacted resin-bound glycine was necessary to prevent nonspecific binding of the enzyme to the resin.

The enzymatic hydrolysis of substrate in solution (Fig. 2) may be compared with resin-bound substrate (as shown in Fig. 3A,B). Overall, the substrate resin in the two-phase system has lower hydrolysis rates than substrates in solution. Figure 3A,B shows the reaction progress curves for the enzymatic hydrolysis of two immobilized substrate analogs where valine and alanine were substituted at the P5 subsite of the resin-bound substrate. The reaction rates of substrate hydrolysis in solution are compared with those of immobilized substrate in Table 2. The COOH-terminal valine analog attached to CLEAR resin demonstrated a slightly higher rate of hydrolysis than the corresponding alanine analog. This contrasting activity is consistent with previous structure function requirements of elastase, where COOH-terminal valine analogs have higher affinity for elastase (12). The rates for substrate hydrolysis are recorded as initial velocities in Table 2. When the initial velocity of the substrate hydrolysis of suc-Ala-Ala-Pro-Val-*p*NA in solution is compared (as shown in Table 2) with its hydrolysis as an immobilized substrate (conjugate I; Fig. 1), the rate of enzymatic release of *p*NA from suc-Ala-Ala-Pro-Val-*p*NA was seven to nine times faster in solution. In contrast, the rate of hydrolysis of suc-Ala-Ala-Pro-Ala-*p*NA was 10–20-fold greater in solution than that attached to resin.

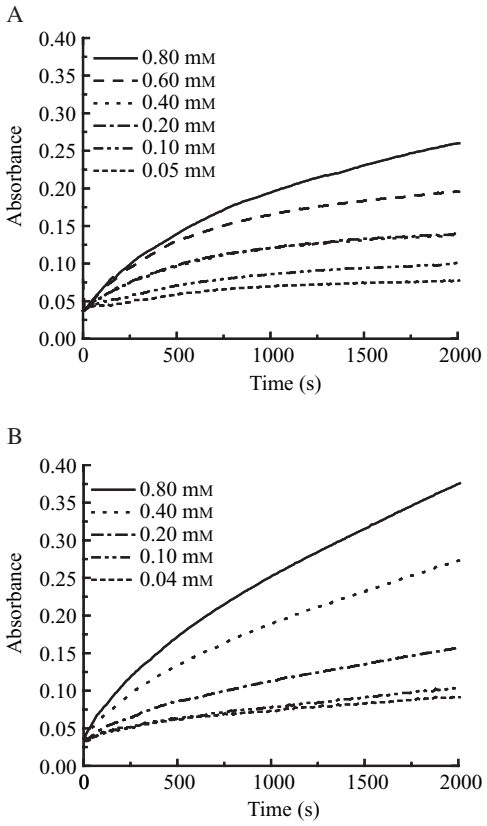

Figure 2. Reaction progress curves of suc-Ala-Ala-Pro-Val-*p*NA and suc-Ala-Ala-Pro-Ala-*p*NA in solution. Reactions were run with the indicated concentration of substrate (40 μL in DMSO) in the presence of 5 μg/mL of elastase as described in the Materials and Methods section. Enzymatic activities of (A) suc-Ala-Ala-Pro-Val-*p*NA and (B) suc-Ala-Ala-Pro-Ala-*p*NA in solution at 405 nm where 60 μL of elastase were placed in each well.

Glycine linkers between the amino-terminal succinylated alanine and the resin polymer were employed to assess the relative effect on enzyme kinetics of conformational space near the resin polymer backbone. A slightly higher rate of substrate hydrolysis was observed with the diglycine spacer between substrate and resin over a single glycine spacer. As seen in Table 2, suc-Ala-Ala-Pro-Ala-*p*NA attached to CLEAR-Gly-Gly gave a twofold increase in rate of hydrolysis compared with the CLEAR-Gly resin analog. It is noteworthy that a longer, more flexible, polar spacer may improve the yield of enzyme cleavage.

Swelling properties of substrate-resin analogs

The swelling properties of substrate resin in phosphate buffer were investigated. The aqueous swelling of modified resins is necessary for availability of enzyme to surface to

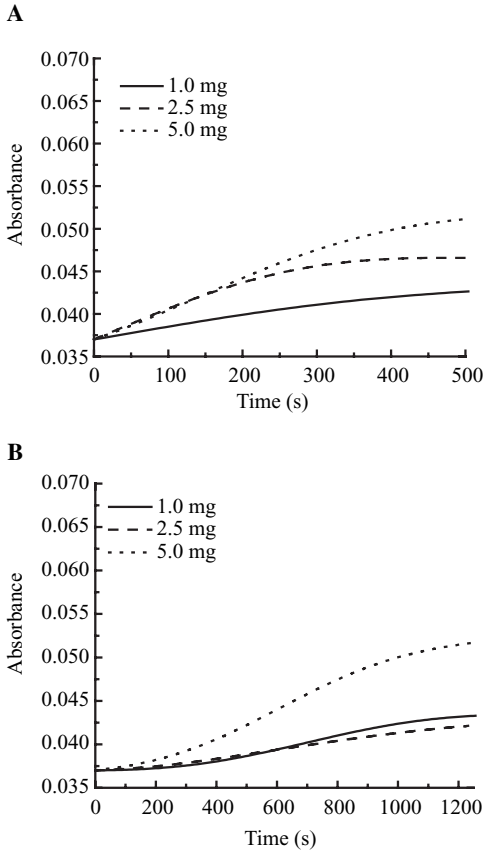

Figure 3. Enzymatic activities of (A) CLEAR-Gly-suc-Ala-Ala-Pro-Val-*p*NA and (B) CLEAR-Gly-suc-Ala-Ala-Pro-Ala-*p*NA at 405 nm where elastase (5 μg/mL) was placed in the well.

resin surface. Swelling of the resin prior to reaction promotes accelerated hydrolysis of the substrate. Figure 4 shows the swelling capacity of the modified resin. The substrate resin swells approximately 1.3 times and 1.7 times more in buffer and DMSO, respectively, than dry, unhydrated resin. The substrate concentration in swollen resin is shown in Table 3. These substrate concentrations are consistent with previous studies, which reported low enzyme binding because of high substrate concentration. Steric hindrance on the polymer surface may abrogate efficient binding at the active site of the enzyme. It is noteworthy that pre-swelling the substrate resin in organic solvent including DCM or DMF followed by solvent exchange to the aqueous buffer may improve the yield of the enzyme cleavage.

Assessment of soluble resin-substrate analogs and polypropylene surface absorption

As increased amounts of resin caused increased light scattering, and interference with the kinetic assessment of resin-substrate hydrolysis in the microtiter plate, the quantitative assessment of chromophore released was performed by chemically releasing a substrate analog from the resin beads followed by assay of the released substrate analog. Thus, the substrate-containing resin was saponified and the released substrate analog was assessed for enzymatic activity. Amino acid analysis of the saponified peptide resin analog revealed quantitative release from the resin. Measurement of the enzymatic reaction progress by absorbance increase of *p*NA from the substrate resin analog was accomplished using 20 mg substrate-resin: 10 μg/mL elastase, 30 mg of substrate resin/20 μg/mL elastase, and 40 mg substrate resin/30 μg/mL elastase. These amounts of resin corresponded to 0.69–4.6 μmol of peptide. The resulting reaction progress curves of the resin analogs of suc-Ala-Ala-Pro-Val-*p*NA and suc-Ala-Ala-Pro-Ala-*p*NA are shown in Fig. 5A–D. The reported absorbance is the average of three measurements. Both analogs showed an increased dose response with increased substrate and enzyme concentrations representative of wound fluid.

Assessment of enzyme activity of the substrate-resin on a surface designed for visible detection of chromophore release was accomplished by assaying the substrate resin on

Table 2. **Concentration of *p*-nitroaniline released from Val analog (P4) and Ala analog (P4)[a]**

Substrate	Concentration (mM) of released *p*NA[b]	Velocity (per second)
Suc-Ala-Ala-Pro-Val-*p*NA (0.8 mM)	6.68E-03	2.50E-04
Immobilized substrate CLEAR-G-suc-AAPV-*p*NA	1.76E-03	3.52E-05
Immobilized substrate CLEAR-GG-suc-AAPV-*p*NA	1.71E-03	2.76E-05
Suc-Ala-Ala-Pro-Ala-*p*NA (0.8 mM)	8.65E-03	3.20E-04
Immobilized substrate CLEAR-G-suc-AAPA-*p*NA	1.72E-03	1.44E-05
Immobilized substrate CLEAR-GG-suc-AAPA-*p*NA	1.63E-03	3.34E-05

a. The concentration of *p*NA released from substrate was obtained by calculating molar absorptivity from a known concentration of para-nitroaniline. Rates were achieved from the slope of the reaction progress curves where absorbance increases over time.
b. Molar absorptivity of *p*NA at concentration of 2.42E–06 M is 2.91E+04 L/mol/cm at 1000 s.

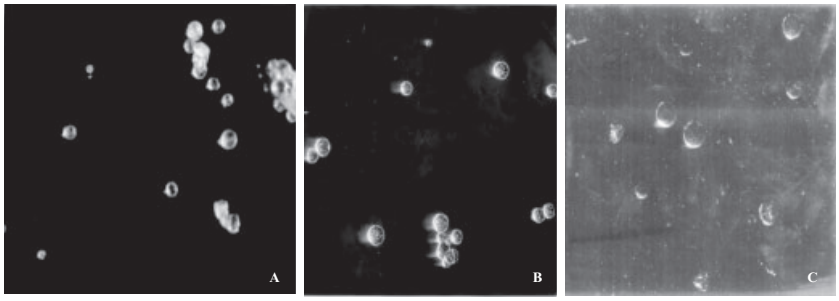

Figure 4. Images of CLEAR-Gly-suc-Ala-Ala-Pro-Val-*p*NA: (A) dry resin; (B) resin in phosphate buffer at pH 7.6; and (C) resin in DMSO (magnification 72×). Measurements of polymer volume were made based on the volume of the swollen modified-CLEAR polymer minus solvent in the void space; approximately 1.3× in phosphate buffer and 1.9× in DMSO.

Table 3. **Ligand concentration in the substrate resin[a]**

Substrate resin	Substrate concentration (μmol/mL) swollen resin
CLEAR-Gly-suc-Ala-Ala-Pro-Val-*p*NA	28.80
CLEAR-Gly-suc-Ala-Ala-Pro-Val-*p*NA	9.80

a. Amino acid analyses were used to determine the substitution level of peptide substrate on the resin. The volume of swollen resin was determined by the difference of the void volume and the settled resin.

a white polypropylene surface. Figure 6 demonstrates results of increased absorption of the substrate-resin chromophore, *p*NA, with time onto a melt-blown polypropylene filter. Both substrate resins demonstrated maximal release of chromophore after 10–20 min. Visible detection of *p*NA accumulation on the polypropylene surface was apparent. The amount of *p*NA deposited on the polypropylene surface was determined based on the absorbance of the plate reader wells. The amount of substrate ligand available for enzy-

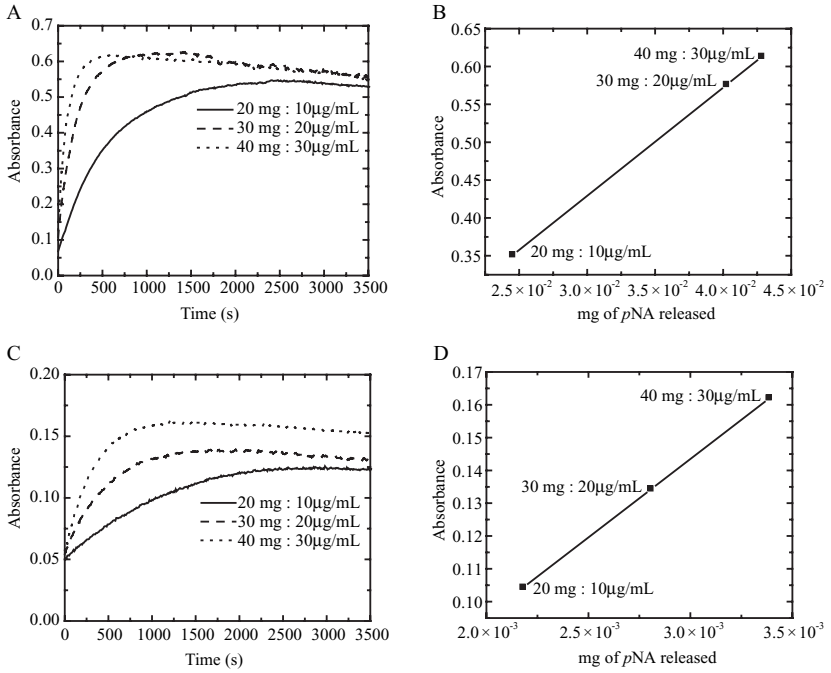

Figure 5. (A) A reaction progress curve of cleaved suc-Ala-Ala-Pro-Val-*p*NA from substrate-CLEAR resin with increasing amounts of 20, 30 and 40 mg combined with 10, 20 and 30 μg/mL of elastase, respectively. (B) A linear increase of absorbance upon enzymatic release of para-nitroaniline with increasing amounts of saponified substrate resin. Substrate-bound resin was treated with 2 M NaOH for 2 h and then centrifuged to separate the supernatant from the cleaved resin as outlined in the materials and methods section. Peptide substrate concentrations of 0.69, 1.03, and 1.38 μmol with respect to increased quantities of resin were combined with elastase where reaction progress was observed at 405 nm. (C) A reaction progress curve of cleaved suc-Ala-Ala-Pro-Ala-*p*NA from CLEAR resin with increasing amounts of 20, 30 and 40 mg combined with 10, 20, and 30 μg/mL of elastase, respectively. (D) A linear increase of absorbance upon enzymatic release of para-nitroaniline with increasing amounts of hydrated resin. Peptide substrate concentrations of 2.30, 3.45 and 4.60 μmol with respect to increased quantities of resin were combined with elastase where the reaction progress was observed at 405 nm.

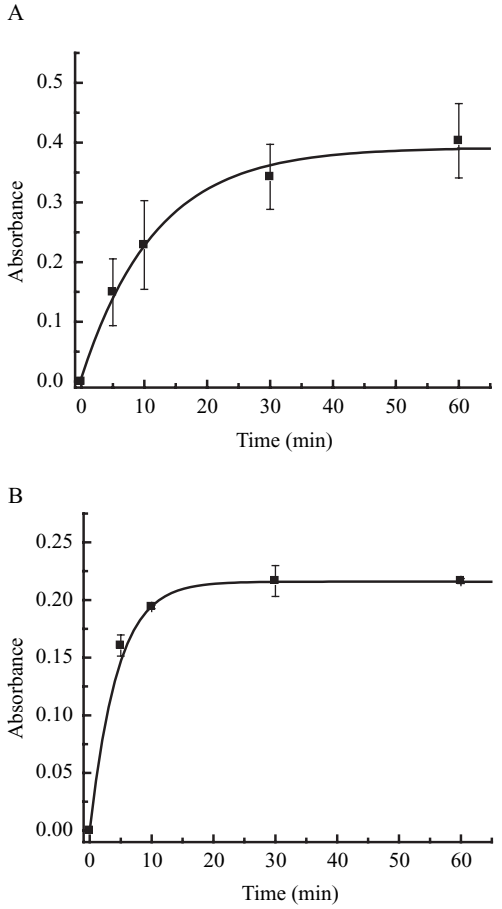

Figure 6. (A) A reaction progress curve of approximately 20 mg of CLEAR-Gly-suc-Ala-Ala-Pro-Val-*p*NA combined with 30 μg/mL of elastase. (B) A reaction progress curve of approximately 20 mg of CLEAR-Gly-suc-Ala-Ala-Pro-Ala-*p*NA combined with 30 μg/mL of elastase. Change in absorbance is a result of chromophore released onto the polypropylene surface with time resulting from enzymatic hydrolysis of substrate as discussed in the Materials and Methods section. Error bars represent one standard deviation of three measurements.

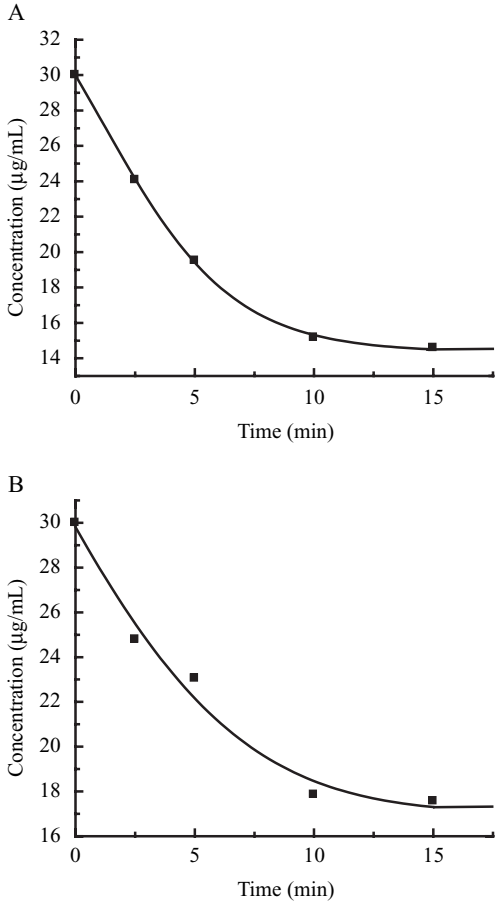

Figure 7. (A) Decrease of elastase concentration in the supernatant of the CLEAR-Gly-suc-Ala-Ala-Pro-Val-*p*NA. (B) Decrease of elastase concentration in the supernatant of the CLEAR-Gly-suc-Ala-Ala-Pro-Ala-*p*NA. The initial elastase concentration was 30 μg/mL. Experiments were carried out in phosphate buffer at pH 7.6 at 25 °C.

matic hydrolysis was 2 and 0.7 μmol in conjugates I and II, respectively. The hydrolysis of conjugate I and conjugate II deposited approximately 40 and 93 nmol on the polypropylene surface, respectively.

Enzyme adsorption by substrate-resin conjugates

The partitioning of elastase between the liquid phase and the resin was assessed. As shown in Fig. 7, an equilibrium state between elastase in solution and resin-adsorbed elastase is established after approximately 20 min. Equilibrium between resin-adsorbed enzyme and enzyme in solution was established within this period. The elastase adsorption by the substrate resin as a function of enzyme concentration partitioning between the two phases was determined. A plot of the equilibrium concentration of enzyme and adsorbed enzyme (Fig. 8) is in accordance with the Langmuir adsorption isotherm as shown.

$$\frac{[E_b]}{[E_g]} = \frac{1}{K_{max}} * [E_b] + \frac{K_a}{K_{max}}$$

where $[E_g]$ is the amount of enzyme per volume of swollen gel, $[E_b]$ is the equilibrium concentration of enzyme in the bulk phase, K_{max} is the maximum adsorption capacity of the substrate resin and K_a is the equilibrium concentration of protein in the bulk phase when the amount of adsorbed enzyme is 50% of the adsorption maximum. Both the K_{max} and the K_a were very low, and the ratio of maximal adsorbed enzyme to bound ligand was <1%. Previous studies of the enzyme hydrolysis of immobilized substrates suggests that a dramatically low protein binding affinity for immobilized substrate may be, in part, a result of high immobilized substrate concentrations (13,14).

Accessibility of the polymer matrix to the enzyme based on molecular size is an important consideration to improve

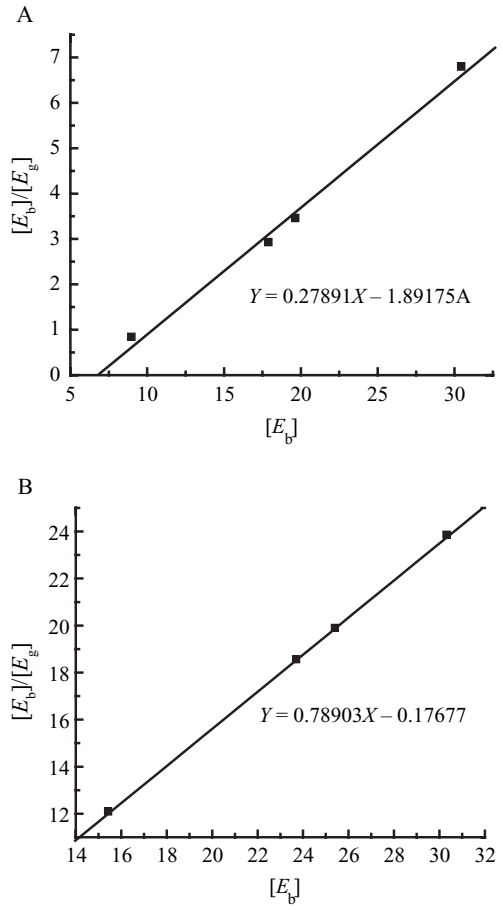

Figure 8. The affinity of elastase to (A) CLEAR-Gly-suc-Ala-Ala-Pro-Val-*p*NA and (B) CLEAR-Gly-suc-Ala-Ala-Pro-Ala-*p*NA substrate resin (K_a) and maximum adsorption capacity of substrate resins (K_{max})

detection sensitivity. Human neutrophil elastase is a 29.5-kDa protease. The resin beads of CLEAR are highly crosslinked and have been shown to contain macropores within a highly dynamic polymer network (10). Thus, the resin beads may accommodate penetration of the protease within the interior of the polymer matrix. The aqueous environment and macroporous nature of the beads may facilitate entry of the enzyme into the polymer bead interior. However, greater resin-bead porosity may facilitate elastase detection sensitivity by making available more enzyme-reactive sites within the resin bead. Previous work on the application of CLEAR beads to biological macromolecules suggests that wide-pore CLEAR supports are permeable to proteins with a fractionation range of 2–660 kDa (15), and low-crosslinked modifications of polyethylene glycol dimethyacrylamide copolymer prepared as immunoaffinity columns have been shown to be permeable to antibodies (150 kDa) (16). Serine protease studies employing solid-phase supports similar to CLEAR, which utilize polyethylene glycol chains within the polymer, have shown varied findings with respect to enzyme penetration of the polymer matrix. For example, Vagner *et al.* (17) showed that proteolysis of peptide substrate on an enzyme-accessible 'surface' area of a polyoxyethylene–polystyrene bead occurs with chymotrypsin while peptides attached to the interior of the bead are not digested. On the contrary, Quarrel *et al.* (18) found that proteins penetrate the resin beads of similar polyoxyethylene beads (TentaGel) completely.

Discussion

Three approaches have been used in assessing elastase activity on the CLEAR-resin-substrate conjugates. The initial approach involved assessment of enzyme activity directly on substrate resin. The relative activities between the immobilized substrate resin and the substrate in solution showed rates from seven to 20 times faster in solution than with substrate resin. However, the kinetic profiles of the substrate resins were minimally measurable under these conditions because of light scattering of the resin beads. Subsequently, the resin substrate conjugate was saponified and the peptide-polymer ester analogs released into solution to obtain a more sensitive measurement of the enzymatic activity of the substrate resin conjugate. Amino acid analysis revealed nearly quantitative release of the peptide ester analogs using saponification of the resin esters to release the intact substrate. The results of the kinetics and chromophore release for the analogs showed that 0.6–28% of the peptide ester analogs reacted with enzyme and underwent substrate hydrolysis. The peptide ester analog of conjugate I demonstrated that 22–28% of available substrate was hydrolyzed; whereas conjugate II demonstrated an average of 0.6% of available substrate hydrolyzed. A third approach to estimating the substrate-resin activity was through assessment of the substrate-resin kinetics on a polypropylene surface. The substrate resin was allowed to react with enzyme on a microtiter-plate polypropylene surface. The amount of chromophore deposited on the polypropylene was found to be 5% of the available substrate.

A polymer matrix containing an immobilized elastase substrate with active properties would be adaptable to a 'dip-stick' type of approach for clinical detection and quantification of the enzyme. The use of immobilized enzyme substrates for the assessment of enzyme activity has received scarce attention compared with the immobilization of enzymes. For example, collagen has been attached to synthetic surfaces as a model to evaluate enzyme-substrate reactions

and diffusion with a surface bound substrate (6). An approach similar to the one discussed in this paper involved chymotrypsin-catalyzed hydrolysis of immobilized substrates using agarose and polyacrylamide-bound substrates of L-phenylalanine 4-nitroanilide (13,14).

The use of an immobilized chromogenic substrate attached to a solid support for elastase detection and quantification may provide a means of conveniently assessing elastase activity in wound fluid through a 'dip-stick' type of approach useful in the clinical detection of the enzyme. CLEAR resin supports swell in both organic and aqueous solvents. This property of amphihilic swelling provides a matrix for incorporation of synthetic enzyme substrates in reasonable yield, which may be assayed in an aqueous phase. The immobilized substrate resin may be assessed for its enzyme activity as a function of its ability to swell in aqueous buffer and gain access to a larger hydrated solvent volume within the polymer in aqueous buffer.

Overall, these three approaches of time dependence of enzymatic hydrolysis, the use of a soluble resin-ester conjugate, and enzymatic release and deposition of the chromophore on a surface, demonstrate both the limits and potential of using a substrate resin to monitor elastase hydrolysis. The limitations of using a minimal amount of substrate resin are shown by the low kinetic response. However, the kinetic profile was enhanced by solubilization of the peptide-substrate esters of the resin. The potential of further developing the substrate resin as a dip-stick method for elastase detection was demonstrated by assay of the resin on a polypropylene surface. However, increased concentrations of chromophore released from enhanced substrate hydrolysis are required to make this approach a concentration sensitive technique. Future studies will focus on increasing the sensitivity of the rate of enzymatic hydrolysis on the resin and tethering the substrate to cellulose materials to visualize elastase activity.

References

1. Janoff, A. (1972) Human neutrophil proteases in inflammation. *Annu. Rev. Med.* **23**, 177–190.
2. Fritz, H., Jochum, M., Duswald, K.H., Dittmer, H., Kortmann, H., Neumann, S. & Lang, H. (1986) Granulocyte proteinases as mediators of unspecific proteolysis in inflammation; a review. In: *Cysteine Proteases and Their Inhibitor* (Turk, V., ed.), pp. 785–807. Walter de Gruyter & Co., Berlin.
3. Yager, D.R., Chan, S.M., Ward, S.I., Olutoye, O.O., Diegelmann, R.F. & Cohen, I.K. (1997) Ability of chronic wound fluids to degrade peptide growth factors is associated with increased levels of elastase activity and diminished levels of proteinase inhibitors. *Wound Repair Regen.* **5**, 23–32.
4. Grinnell, F. & Zhu, M. (1994) Identification of neutrophil elastase as the proteinase in burn wound fluid responsible for degradation of fibronectin. *J. Invest. Dermatol.* **103**, 155–161.
5. Clark, R.A.F. & Henson, P.M. (1998) *The Molecular and Cellular Biology of Wound Repair*. Plenum, New York.
6. Edwards, J.V., Bopp, A.F., Batiste, S., Ullah, A.J., Cohen, I.K., Diegelmann, R.F. & Montante, S.J. (1999) Inhibition of elastase by a synthetic cotton bound serine protease inhibitor: in vitro kinetics and inhibitor release. *Wound Repair Regen.* **7**, 106–118.
7. Edwards, J.V., Yager, D.R., Cohen, I.K., Diegelmann, R.F., Montante, S., Bertoniere, S.N. & Bopp, A.F. (2001) Modified cotton gauze dressings that selectively absorb neutrophil elastase activity in solution. *Wound Repair Regen.* **9**, 50–58.
8. Cullen, B., Smith, R., McCulloch, E., Silcock, D., Morrison, L. (2002) Mechanism of action of PROMOGRAN, a protease modulating matrix, for the treatment of diabetic foot ulcers. *Wound Repair Regen.* **10**, 16–25.
9. Gaspers, P.B., Gast, A.P. & Robertson, C.R. (1995) Enzymes on immobilized substrate surfaces: reaction. *J. Colloid Interface Sci.* **172**, 518–529.
10. Kempe, M. & Barany, G. (1996) CLEAR: A novel family of highly cross-linked polymeric supports for solid-phase peptide synthesis. *J. Am. Chem. Soc.* **118**, 7083–7093.
11. Kaiser, E., Colescott, R.L., Bossinger, C.D. & Cook, P.I. (1970) Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* **34**, 595–598.
12. Nakjima, K., Powers, J.C., Ashe, B.M. & Zimmerman, M. (1979) Mapping the extended substrate binding site of cathepsin G and human leukocyte elastase. *J. Biol. Chem.* **254**, 4027–4032.
13. Fischer, J., Lange, L. & Jakubke, H.-D. (1978) Enzymatic attack on immobilized substrates 2. Diffusional limitations in the α-chymotrypsin-catalyzed hydrolysis of polyacrylamide-bound L-phenylalanine 4-nitroanilide. *Eur. J. Biochem.* **88**, 453–457.
14. Fischer, J., Lange, L., Koch, S. & Jakubke, H.-D. (1978) Enzymatic attack on immobilized substrates 1. The α-chymotrypsin-catalzyed hydrolysis. *Eur. J. Biochem.* **88**, 445–452.
15. Cederfur, J. & Kempe, M. (2001) Development of wide-pore CLEAR supports for applications involving biological macromolecules. *Polymer Bull.* **46**, 381–387.
16. Auzanneau, F.-I., Christensen, M.K., Harris, S.L., Meldal, M. & Pinto, B.M. (1998) Synthesis and characterization of polyethylene glycol polyacrylamide copolymer (PEGA) resins containing carbohydrate ligands. Evaluation as supports for affinity chromatography. *Can. J. Chem.* **76**, 1109–1118.
17. Vagner, J., Barany, G., Lam, S.K., Krchnak, V., Septeov, N.F., Ostrem, J.A., Strop, P. & Lebl, M. (1996) Enzyme-mediated spatial segregation on individual polymeric support beads: application to generation and screening of encoded combinatorial libraries. *Proc. Natl. Acad. Sci. USA* **93**, 8194–8199.
18. Quarrell, R., Claridge, T.D.W., Weaver, G.W. & Lowe, G. (1996) Structure and properties of Tentagel resin beads: implications for combinatorial library chemistry. *Mol. Diversity* **1**, 223–232.